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(54) Title: MODULATION OF PLATELET ACTIVATION

(57) Abstract: Methods of modulating thrombin-mediated platelet activation by inhibition or enhancement of protease-activated receptor 4(PAR4) and protease-activated receptor 1(PAR1) activity are disclosed. This method provides a way of substantially blocking all thrombin-mediated activation of platelets by 1) inhibiting signalling though PAR1 and 2) inhibiting signalling through PAR4. Signalling through each PAR receptor may be inhibited at various molecular levels, including: the level of ligand binding (*e.g.* by administration of an antagonist), the level of the receptor activity (*e.g* blocking expression of the receptor in the relevant cells) and/or intracellularly, (*e.g.* blocking expression or activity of a molecule required for activity of the receptor). Alternatively, this method provides a method for enhancing thrombin-mediated platelet activation by specifically activating PAR1 and PAR4. These methods may take place *in vivo*, through administration of the appropriate compounds, or *in vitro*, *e.g.* the *ex vivo* treatment of a sample.

MODULATION OF PLATELET ACTIVATION

FIELD OF THE INVENTION

This invention relates to inhibition of platelet activation, and particularly to such inhibition mediated through thrombin receptors.

BACKGROUND OF THE INVENTION

Thrombin, a coagulation protease generated at sites of vascular injury, activates platelets, leukocytes, and mesenchymal cells (T.-K.H. Vu *et al., Cell* 64:1057-1068 (1991)). Activation of platelets by thrombin is thought to be critical for hemostasis and thrombosis. In animal models, thrombin inhibitors block platelet-dependent thrombosis, which is the cause of most heart attacks and strokes in humans. Available data in humans suggests that thrombosis in arteries can be blocked by inhibitors of platelet function and by thrombin inhibitors. Thus it is likely that thrombin's actions on platelets contribute to the formation of clots that cause heart attack and stroke. Thrombin's other actions on vascular endothelial cells and smooth muscle cells, leukocytes, and fibroblasts may mediate inflammatory and proliferative responses to injury, as occur in normal wound healing and a variety of diseases (atherosclerosis, restenosis, pulmonary inflammation (ARDS), glomerulosclerosis, etc.).

Thrombin signaling is mediated at least in part by a family of G protein-coupled protease-activated receptors (PARs) for which PAR1 is the prototype. U. B. Rasmussen *et al., FEBS Letts.* 288:123-128 (1991). PAR1 is activated when thrombin binds to and cleaves its amino terminal exodomain to unmask a new receptor amino terminus. This new amino terminus then serves as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect transmembrane signaling. T.-K. Vu *et al., Nature.* 353:674-677 (1991); J. M. Chen *et al., J. Biol. Chem.*, 269:16041-16045 (1994). The synthetic peptide SFLLRN, which mimics the first six amino acids of the new amino terminus unmasked by receptor cleavage, functions as a PAR1 agonist and activates the receptor independent of thrombin and proteolysis. R. J. Vassallo *et al., J. Biol. Chem.*, 267:6081-6085 (1994).R. M. Scarborough *et al., J. Biol Chem.* 267:13146-9 (1992). Such peptides have been used as pharmacological probes of PAR function in various cell types.

Our understanding of the role of PARs in platelet activation is evolving rapidly. PAR1 mRNA and protein were detected in human platelets. D. T. Hung et al., *J.Clinical*

Investigation. 89:1350-3 (1992); L. F. Brass et al., *J. Biol. Chem.* 267:13795-13798 (1992); M. Molino et al., *J. Biol Chem.* 272:6011-7 (1997). SFLLRN activated human platelets 3, 7, 8, and PAR1-blocking antibodies inhibited human platelet activation by low, but not high concentrations of thrombin These data suggest a role for PAR1 in activation of human platelets by thrombin but hold open the possibility that other receptors contribute. PAR4 was recently identified. M. L. Kahn, *Nature*. 394:690-694 (1998); W. F. Xu *et al., PNAS* 95:6642-6 (1998), and appears to function in both mouse and human platelets.

There is a need for a better understanding of thrombin-mediated platelet activation. There is also a need for the identification and characterization of factors that may either contribute to or inhibit platelet-mediated pathologies such as platelet-dependent arterial thrombosis for use in treating associated pathologies.

SUMMARY OF THE INVENTION

Methods of modulating thrombin-mediated platelet activation by inhibition or enhancement of protease-activated receptor 4 (PAR4) and protease-activated receptor 1 (PAR1) activity are disclosed. This method provides a way of substantially blocking all thrombin-mediated activation of platelets by 1) inhibiting signaling through PAR1 and 2) inhibiting signaling through PAR4. Signaling through each PAR receptor may be inhibited at various molecular levels, including: the level of ligand binding (*e.g.* by administration of an antagonist), the level of the receptor activity (*e.g.* blocking expression of the receptor in the relevant cells) and/or intracellularly, (*e.g.* blocking expression or activity of a molecule required for activity of the receptor). Alternatively, this method provides a method for enhancing thrombin-mediated platelet activation by specifically activating PAR1 and PAR4. These methods may take place *in vivo*, through administration of the appropriate compounds, or *in vitro*, *e.g.* the *ex vivo* treatment of a sample.

The present invention provides compositions that are effective for prevention of thrombin-associated platelet activation. These compositions are comprised of agents that inhibit the activity of PAR1 and PAR4, *e.g.* antagonists of PAR1 and PAR4, and are effective in the treatment of disorders. Examples of useful antagonists are small molecules, modeled proteins, and antibodies. In addition, compositions may comprise dominant negative PAR receptors, which may be employed to substantially decrease or eliminate the

expression of either PAR1 or PAR4. These compositions may also contain nucleic acid sequences that encode for antagonists of PAR1 and PAR4, which may be administered for expression *in vivo*.

The present invention also provides compositions that are effective for enhancing thrombin-associated platelet activation, either *in vitro* and/or *in vivo*. These compositions are comprised of agents that inhibit the activity of both PAR1 and PAR4, *e.g.* antagonists of PAR1 and PAR4, and are effective in the treatment of disorders. Examples of useful antagonists are small molecules, modeled proteins, and antibodies. These compositions may also contain nucleic acid sequences that encode for antagonists of PAR1 and PAR4, which may be administered for expression *in vivo*. These compositions are comprised of agents that enhance the activity of both PAR1 and PAR4, *e.g.* antagonists of PAR1 and PAR4, and are effective in the treatment of disorders wherein there is insufficient activation of platelets, *e.g.* hemophilia. Examples of useful agonists are small molecules, modeled proteins, and antibodies. These compositions may also contain nucleic acid sequences that encode for antagonists of PAR1 and PAR4, which may be administered for expression *in vivo*.

In one embodiment, the invention provides therapeutic uses of the inhibiting compositions of the invention in the treatment of disorders such as such as myocardial infarction, stroke, pulmonary embolism, deep vein thrombosis, peripheral arterial occlusion, and other blood system thromboses. One method of treatment comprises the administration of protonated/acidified nucleic acids to the animal in an amount sufficient to inhibit or prevent tissue occlusion. Alternatively, a sample may be taken from an animal (*e.g.* a blood sample), treated *ex vivo* with the inhibiting composition of the invention, and returned to the animal.

In another embodiment, the invention provides therapeutic uses of the activating compositions of the invention in disorders involving insufficient clotting. The dual activation of PAR1 and PAR4 may increase the activation of platelets, since thrombin has the ability to activate both receptors.

The invention also provides specific PAR4 antibodies for use in the methods of the invention. Such antibodies effectively block signaling through PAR4 and thus effectively block PAR4's contribution to thrombin-mediated platelet activation.

It is an object of the invention to inhibit activity of PAR1 and PAR4, thus substantially eliminating thrombin mediated platelet activation.

It is an advantage of the invention that the method can be practiced both *in vivo* and *ex vivo*.

It is a feature of the invention that the pharmaceutical compositions of the invention are effective at treating a variety of ailments.

These and other objects, advantages, and features of the invention will become apparent to those skilled in the art upon reading the details of the nucleic acids and uses thereof as more fully described below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is the summary of studies of a competitive RT-PCR assay of RNA prepared from platelets, neutrophils and Dami cells.

Figure 2 is a graph illustrating antibody binding to the surface of receptor-expressing COS cells.

Figures 3A - 3D is a series of graphs illustrating the results of flow cytometric analysis of human platelets.

Figures 4A - 4D is a series of graphs illustrating the results of flow cytometric analysis of Dami cells.

Figure 5 illustrates the ability of the PAR1 and PAR4 tethered ligand peptides to activate PAR1 and PAR4 heterologously expressed in *Xenopus* oocytes.

Figures 6A - 6C illustrate the ability of the PAR1 and PAR4 tethered ligand peptides to activate PAR1 and PAR4 human platelets.

Figure 7 is a graph illustrating the ability of the PAR1 and PAR4 antibodies to block thrombin cleavage of PAR1 and PAR4in rat I fibroblasts expressing FLAG epitope-tagged PAR1 and PAR4.

Figures 8A and 8B illustrate the increases in cytoplasmic calcium in response to thrombin in cells expressing PAR 1 (Figure 8A) or PAR 4 (Figure 8B).

Figures 9A - 9E illustrate the contribution of PAR1 and PAR4 signaling to thrombin activation of human platelets with and without treatment with PAR activation peptides or antibodies.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Before the present protease-activated receptor assays and methods of using such are described, it is to be understood that this invention is not limited to the particular DNA sequences, materials, methods, or processes described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

It must be noted that as used in this specification and the appended claims, the singular forms "a", "and," and "the" include plural referents unless the contexts clearly dictates otherwise. Thus, for example, reference to "a DNA sequence" includes mixtures and large numbers of such sequences, reference to "an assay" includes assays of the same general type, and reference to "the method" includes one or more methods or steps of the type described herein.

The publications discussed herein are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

Unless defined otherwise, all technical and scientific terms herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials, similar or equivalent to those described herein, can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications cited herein are incorporated herein by reference for the purpose of disclosing and describing specific aspects of the invention for which the publication is cited in connection with.

DEFINITIONS

By "protease-activated receptor 1", "PAR1", "PAR1 receptor" and the like, is meant all or part of a vertebrate cell surface protein which is specifically activated by thrombin or a thrombin agonist thereby activating PAR1-mediated signaling events. The polypeptide is characterized as having the properties (including the agonist activating and antagonist inhibiting properties) described herein and in Vu et al. (1991) *Cell*, 64:1057-1068, which is incorporated herein by reference. PAR1 may refer to a naturally occurring form of the

receptor and/or a recombinantly produced form of the receptor. In addition, the term may include variants of the PAR1 protein that retain the same activity and properties.

By "protease-activated receptor 4", "PAR4", "PAR4 receptor" and the like, is meant all or part of a vertebrate cell surface protein which is specifically activated by thrombin or a thrombin agonist thereby activating PAR4-mediated signaling events. The polypeptide is characterized as having the properties (including the agonist activating and antagonist inhibiting properties) described herein and in U.S. Application No. 09/032,397, which is incorporated herein by reference. PAR4 may refer to a naturally occurring form of the receptor and/or a recombinantly produced form of the receptor. In addition, the term may include variants of the PAR4 protein that retain the same activity and properties.

By a "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (*e.g.*, glycosylation).

By "substantially pure" is meant that the protease-activated receptor 4 polypeptide provided by the invention is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, PAR polypeptide. A substantially pure PAR polypeptide may be obtained, for example, by extraction from a natural source, by expression of a recombinant nucleic acid encoding a PAR polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, *e.g.*, column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis. The protein is substantially pure if it can be isolated to a band in a gel.

By a "substantially identical" amino acid sequence is meant an amino acid sequence which differs only by conservative amino acid substitutions, for example, substitution of one amino acid for another of the same class (*e.g.*, valine for leucine, arginine for lysine, etc.) or by one or more non-conservative amino acid substitutions, deletions, or insertions located at positions of the amino acid sequence which do not destroy the biological activity of the receptor. Such equivalent receptors can be isolated by extraction from the tissues or cells of any animal which naturally produces such a receptor or which can be induced to do so, using the methods described below, or their equivalent; or can be isolated by chemical synthesis; or can be isolated by standard techniques of recombinant DNA technology, *e.g.*, by isolation of cDNA or genomic DNA encoding such a receptor. Substantially identical receptors have the same biological function, *e.g.* are activated by the same compound.

By "derived from" is meant encoded by the genome of that organism and present on the surface of a subset of that organism's cells.

By "isolated DNA" is meant DNA that is not in its native environment in terms of not being immediately contiguous with (*i.e.*, covalently linked to) the complete coding sequences with which it is immediately contiguous (*i.e.*, one at the 5' end and one at the 3' end) in the naturally-occurring genome of the organism from which the DNA of the invention is derived. The term therefore includes, for example, recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote; or which exists as a separate molecule (*e.g.*, a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes any recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "transformed cell" "transfected cell", "genetically engineered cell", and the like, is meant a cell into which (or into an ancestor of which) has been introduced, by means of genetic engineering, a DNA molecule encoding PAR1, PAR4 or a biologically active fragment of either. Such a DNA molecule is "positioned for expression" meaning that the DNA molecule is positioned adjacent to a DNA sequence which directs transcription and translation of the sequence (*i.e.*, facilitates the production of a PAR1 or PAR4 protein, or fragment or analog thereof).

By "antibody" is meant an immunoglobulin protein which is capable of binding an antigen. Antibody as used herein is meant to include the entire antibody as well as any antibody fragments (*e.g.* $F(ab')_2$, Fab', Fab, Fv) capable of binding the epitope, antigen or antigenic fragment of interest.

Antibodies of the invention are immunoreactive or immunospecific for and therefore specifically and selectively bind to either PAR1 or PAR4 protein. Antibodies for PAR1 or PAR4 are preferably immunospecific -- *i.e.*, not substantially cross-reactive with related materials, *e.g.*, with each other. Although the term "antibody" encompasses all types of antibodies (*e.g.*, monoclonal) the antibodies of the invention are preferably produced using the phage display methodology described herein. The preferred antibody of the invention is a purified antibody. By purified antibody is meant one which is sufficiently free of other proteins, carbohydrates, and lipids with which it is naturally associated. Such an antibody

"preferentially binds" to a specific PAR protein (or an antigenic fragment thereof), *i.e.*, does not substantially recognize and bind to other antigenically-unrelated molecules.

By "specifically activates", as used herein, is meant an agent, such as thrombin, a thrombin analog, a PAR agonist or other chemical agent including polypeptides such as an antibody, which activates protease-activated receptor, receptor polypeptide or a fragment or analog thereof to initiate PAR-mediated biological events as described herein, but which does not substantially bind other molecules in a sample, *e.g.*, a biological sample, which naturally includes a protease-activated receptor polypeptide.

By "specifically inhibits", as used herein, is meant an agent, such as a thrombin analog, a PAR4 antagonist or other chemical agent including polypeptides such as an antibody, which inhibits activation of protease-activated receptor 4, receptor polypeptide or a fragment or analog thereof, such as by inhibiting thrombin or by blocking activation of PAR4 by thrombin or other PAR4 activator. Preferably, the agent activates or inhibits the biological activity *in vivo* or *in vitro* of the protein to which it binds.

By "biological activity" is meant the ability of the PAR to bind thrombin or an agonist and signal the appropriate cascade of biological events (*e.g.*, phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation, and the like).

By "substantial increase" is meant an increase in activity or other measurable phenotypic characteristic that is at least approximately a 2-fold increase over control level (where control assays are performed in the absence of activator), preferably at least approximately a 5-fold increase, more preferably at least approximately a 10-fold increase in activity over a control assay.

By "substantial decrease" or "substantial reduction" is meant a decrease or reduction in activity or other measurable phenotypic characteristic that is approximately 80% or the control level, preferably reduced to approximately 50% of the control level, or more preferably reduced to approximately 10% or less of the control level.

The terms "screening method" and "assay method" are used to describe a method of screening a candidate compound for its ability to act as an agonist or antagonist of a PAR4 ligand. The method involves: a) contacting a candidate agonist compound with a recombinant protease-activated receptor 4 (or PAR4 agonist-binding fragment or analog); b) measuring activation of the receptor, the receptor polypeptide or the receptor fragment or analog; and c) identifying agonist compounds as those which interact with the recombinant

receptor and trigger or block PAR4 activation. Interaction may be cleavage of the receptor to unmask an intramolecular receptor activating peptide or by mimicking the intramolecular receptor-activating peptide. A tethered ligand may be more difficult to block than a free agonist. Thus, blocking thrombin is the acid test for an antagonist which will block responses by other thrombin substrates. These terms include assays that examine effects on unoccupied receptors as well as assays that utilize displacement of a ligand from an occupied receptor.

By an "agonist" is meant a molecule which mimics a particular activity, in this case, interacting with a PAR or PAR ligand in a manner which activates thereby triggering the biological events which normally result from the interaction (*e.g.*, phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation). Preferably, an agonist initiates a substantial increase in receptor activity relative to control assays in the absence of activator or candidate agonist. An agonist may possess the same, less, or greater activity than a naturally-occurring PAR ligand.

By an "antagonist" is meant a molecule which blocks activation of a PAR receptor. This can be done by inhibiting a particular activity such as the ability of thrombin, for example, to interact with a PAR thereby triggering the biological events resulting from such an interaction (*e.g.*, phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet secretion, or platelet aggregation). An antagonist may bind to and thereby block activation of a PAR receptor, either PAR1, PAR4 or both.

The terms "antagonist assay", "antagonist screening" and the like, refer to a method of screening a candidate compound for its ability to antagonize interaction between a naturally-occurring activating ligand or an agonist and the PAR, either PAR1, PAR 4 or both. The method involves: a) contacting a candidate antagonist compound with a first compound which includes a recombinant PAR (or agonist-binding fragment or analog) on the one hand and with a second compound which includes thrombin or a PAR agonist on the other hand; b) determining whether the first and second compounds interact or are prevented from interaction by the candidate compound; and c) identifying antagonistic compounds as those which interfere with the interaction of the first compound (PAR receptor) to the second compound (PAR agonist) and which thereby substantially reduce thrombin or PAR agonist-activated biological events (*e.g.*, phosphoinositide hydrolysis, Ca^{2+} efflux, and platelet aggregation).

The terms "treatment", "treating", "treat" and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particular a human, and includes:

(a) preventing the disease or symptom from occurring in a subject which may be predisposed to the disease or symptom but has not yet been diagnosed as having it;

(b) inhibiting the disease symptom, *i.e.*, arresting its development; or

(c) relieving the disease symptom, *i.e.*, causing regression of the disease.

The term "extracorporeal blood" includes blood removed in line from a patient, subjected to extracorporeal treatment, and returned to the patient in processes such as dialysis procedures or blood filtration or blood bypass during surgery. The term also includes blood products which are stored extracorporeally for eventual administration to a patient. Such products include whole blood, platelet concentrates and any other blood fraction in which inhibition or enhancement of platelet aggregation and platelet release is desired.

GENERAL ASPECTS OF THE INVENTION

The present invention is based upon the discovery that inhibition of PAR1 and PAR4 virtually abolishes the ability of platelets to respond to thrombin. Functional studies with PAR1 and PAR4 activating peptides confirmed that activation of either receptor was sufficient to trigger platelet secretion and aggregation. Given thrombin's remarkable potency as a platelet activator, blocking thrombin signaling in platelets is useful in any number of biological phenomena, such as prevention of thrombosis.

The invention thus provides a method of treatment for reducing the level of thrombin response in a mammalian host by administering a composition which inhibits both PAR1 and PAR4 activity. In general, such compounds will reduce the activity of the PAR1 and PAR4 receptors. Individuals treated may not presently exhibit symptoms, but prophylactic use is contemplated for individuals at risk for disorders such as thromboembolism, such as the elderly and/or individuals with a history of problems with thrombi (*i.e.* blood clots).

A number of mechanisms can be used to selectively block PAR1 signaling. For example, peptides that selectively bind to and inactivate PAR1 may be used to block PAR1 signaling. The availability of a potent and selective PAR1 peptide agonist allows for homologous desensitization of platelets to PAR1 activation. In another example, antibodies that selectively bind PAR1, but not the other PARs, can be used, *e.g.* antibodies to PAR1's hirudin-like domain, which inhibit PAR1 cleavage and activation by thrombin. In a third example, PAR1-specific antagonists permit direct blockade of PAR1 signaling, while leaving the signaling through the other PAR molecules generally unaffected. Each of these examples, as well as others known by those skilled in the art, may be used to inhibit PAR1 signaling without interfering with other PAR function. Inhibition of PAR1 function by antibody, antagonist, or desensitization markedly inhibits platelet responses at low (1nM) concentrations of thrombin.

PAR4 may be inhibited using similar mechanisms, including but not limited to peptide agonists, antibodies, and PAR4-specific antagonists. PAR4 inhibition is preferably accomplished using an antibody to PAR4's thrombin cleavage site. This antibody preparation blocks PAR4 activation by thrombin without interfering with other PAR activity. Moreover, an antibody to this region of PAR4 does not inhibit activation of either PAR4 or platelets by the PAR4-activating peptide GYPGKF. In contrast to PAR1 inhibition, inhibition of PAR4 alone had no significant effect on platelet aggregation.

The treatment of the present invention may take place *in vivo*, *e.g.* through the administration of PAR1 and PAR4 antagonists or introduction and expression of PAR1 and PAR4 antagonists. Alternatively, the treatment may take place *ex vivo*, *e.g.* a patient's extracorporeal blood may be treated with compositions of the invention and the blood replaced.

COMPOUNDS OF THE INVENTION

Compounds of the invention encompass numerous chemical classes, including but not limited to the compounds described herein with known function.

Novel methods are provided which employ compounds that are effective in decreasing the level of PAR1 and PAR4 in mammalian cells.

Candidate compounds can be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available

for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological compounds may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, *etc.* to produce structural analogs.

The agonists or antagonists of the present invention may relate to synthetic polypeptides that bind to of PAR1 and/or PAR4. Such synthetic antiplatelet polypeptides may be prepared by conventional chemical synthesis techniques, for example, synthesis on a solid support.

The present invention also relates to recombinant and synthetic DNA molecules which encode molecules that either inhibit or enhance PAR activity. The synthesis of these DNA molecules may be achieved by methods well known in the art. For example, the recombinant DNA molecules may be isolated from a human hematopoetic cDNA library. The synthesis of cDNA libraries and the choice of vector into which the cDNA molecules may be cloned are conventional techniques, *see e.g.* T. Maniatis et al., "Molecular Cloning -- A Laboratory Manual", Cold Spring Harbor (1982). A wide variety of methods may be used in locating and identifying cDNA sequences corresponding to a the compositions of the present invention. The two most preferred techniques are the use of oligonucleotide probe based on the amino acid sequence of the of PAR1 or PAR4 ligands, and immunoscreening, which utilizes antibodies against the extracellular domains of PAR1 and PAR4 to detect clones which express cDNA sequences corresponding to potential agonist or antagonist activity. It will be obvious to those of skill in the art that the choice of oligonucleotides probes will be based upon those strengths of amino acids which are encoded by the least redundant DNA sequences.

The immunoscreening technique requires that the cDNA library be contained in a vector capable of expression. Such vectors include lambda gt11, lambda gt10 and other expression vectors known in the art. Antibodies employed in the immunoscreening technique include antibodies against intact PAR1 and PAR4, antibodies against denatured PAR1 and

PAR4 and antibodies against peptide portions of PAR1 and PAR4. Once a potentially interesting cDNA has been identified and isolated, it may be transcribed and translated to determine whether it encodes a peptide with PAR antagonist or agonist activity. Partial cDNAs may themselves be used to reprobe the cDNA library and to locate full-length cDNAs.

Alternatively, the DNA molecules of this invention may be synthesized from nucleotides by chemical means using an synthesizer. Such nucleic acids may be designed based on identified amino acid sequence of the PAR agonists or antagonists. Standard methods may be applied to synthesize a gene encoding such a peptide. For example, the complete amino acid sequence may be used to construct a back-translated gene. A DNA oligomer containing a nucleotide sequence capable of coding for the desired polypeptide may be synthesized in a single step.

Alternatively, several smaller oligonucleotides coding for portions of the PAR1 and/or PAR4 agonist or antagonist may be synthesized and subsequently ligated together. Preferably, the antiplatelet polypeptide gene is synthesized as 10-20 separate oligonucleotides which are subsequently linked together. The individual oligonucleotides contain 5' or 3' overhangs for complementary assembly.

Following synthesis of the nucleic acid and cleavage of the desired vector, assembly of the antiplatelet polypeptide gene may be achieved in one or more steps by techniques well known in the art. Once assembled, the gene will be characterized by sequences which are recognized by restriction endonucleases, including unique restriction sites for direct assembly into a cloning or an expression vector; preferential codons based upon the host expression system to be used: and a sequence which, when transcribed, produces an mRNA with minimal secondary structure. Proper assembly may be confirmed by nucleotide sequencing, restriction mapping, and expression of a biologically active antagonist or agonist in a platelet aggregation assay.

According to one embodiment, the present invention relates to compositions for decreasing or preventing platelet aggregation and release and methods which employ them. These compositions may also contain a variety of other conventional antiplatelet or anti-thrombin compounds in addition to a naturally purified, recombinant or synthetic polypeptide inhibitor of platelet activation of this invention. The most widely used antiplatelet agent is aspirin, a cyclooxygenase inhibitor. Although aspirin blocks ADP-

and collagen-induced platelet aggregation, it fails to prevent cyclooxygenase-independent platelet aggregation initiated by agonists, such as thrombin. Alternative anti-thrombin compounds are hirudin derivatives.

The composition of the invention containing additional anti-platelet activation compounds may be a single dosage form, wherein a polypeptide inhibitor of platelet activation of this invention may be chemically conjugated to a conventional polypeptide platelet inhibitor or to a conventional anti-thrombin polypeptide. Alternatively, a single dosage form which contains the polypeptide inhibitor of platelet activation and the other polypeptide in the same composition, but as separate compounds. The composition may also contain multiple dosage forms, wherein the PAR1 and PAR4 inhibitors and the other polypeptide that inhibits of platelet activation are administered separately, but concurrently, or wherein the two forms are administered sequentially.

A polypeptide inhibitor of PAR1 and/or PAR4 may also be cross-linked to a conventional carrier polypeptide, which may be carried out by chemical cross-linking methods well known in the art. Most preferably, such combinations are formed by cross-linking a natural or recombinant polypeptide PAR antagonists to carriers that have been synthesized with a cross-linking moiety, such as dinitrofluorobenzene, at its NH_2 terminus. Alternatively, the carrier peptide may be conjugated to a natural or recombinant PAR 1 and/or PAR4 antagonist by the use of agents such as glutaraldehyde, dimethyladipimidate, or any other bifunctional cross-linkers known in the art. The conjugated antagonist preferably involves a 1:1 stoichiometry with the carrier peptide.

The agonists or antagonists of the present invention may be present invention relates to synthetic polypeptides of platelet activation. Such synthetic antiplatelet polypeptides may be prepared by conventional chemical synthesis techniques, for example, synthesis on a solid support.

Methods of generating antibodies that may be used in the methods of the present invention are generally known to those skilled in the art. For example, antibodies detecting the extracellular domain of either PAR1 or PAR4 may be generated by immunizing rabbits or mice with a portion of the extracellular domain of each molecule, or a peptide fragment derived therefrom. Only antibodies with affinity at least 4 fold higher for PAR1 or PAR4, respectively as compared to their affinity for any other PAR molecules should be selected. The method of antibody generation, purification, labeling and detection may vary.

The IgG or Fab's may be purified from different sources by affinity HPLC using protein A column and Size exclusion HPLC. The purified antibodies may be labeled with Europium and detected by time resolved fluorescence. The antibody binding to different PAR proteins may be measured by time-resolved, dissociation-enhanced fluorescence. However, the system of detection of PAR-bound IG on solid support *in situ* or in solution may vary. Further, it is possible to use direct or indirect immunological methods including direct radiolabels, fluorescence, luminescence, avidin-biotin amplification, or enzyme-linked assays with color or luminescent substrates. The preferred PAR1 antibody which can be used in the invention is disclosed in Hung et al. (1992) *J. Clin. Invest.*, 89:1350-1353.

For purposes of the invention an indication that no binding occurs means that the equilibrium or affinity constant K_a is 10^6 l/mole or less. Further, binding will be recognized as existing when the K_a is at 10^7 l/mole or greater, preferably 10^8 l/mole or greater. The binding affinity of 10^7 l/mole or more may be due to (1) a single monoclonal antibody (*i.e.*, large numbers of one kind of antibodies) or (2) a plurality of different monoclonal antibodies (*e.g.*, large numbers of each of five different monoclonal antibodies) or (3) large numbers of polyclonal antibodies. It is also possible to use combinations of (1) - (3). Selected preferred antibodies will bind at least 4-fold more avidly to one PAR (*e.g.* PAR1) than to a different PAR (*e.g.* PAR4). The four fold differential in binding affinity may be accomplished by using several different antibodies as per (1) - (3) above and as such some of the antibodies in a mixture could have less than a four fold difference.

The methods of the present invention may be practiced using one or more different antibodies to PAR1 and/or PAR4. Those skill in the art will recognize that antibodies may be labeled with known labels and used with currently available robotics, sandwich assays, electronic detectors, flow cytometry, and the like.

ADMINISTRATION OF THE COMPOUNDS

In the subject methods, the compound may be administered to the host using any convenient means capable of resulting in the desired target protein activity modulation. Thus, the compound can be incorporated into a variety of formulations for therapeutic administration. More particularly, the compounds of the present invention can be formulated into pharmaceutical compositions by combination with appropriate, pharmaceutically acceptable carriers or diluents, and may be formulated into preparations

in solid, semi-solid, liquid or gaseous forms, such as tablets, capsules, powders, granules, ointments, solutions, transdermal patches, suppositories, injections, inhalants and aerosols.

As such, administration of the compounds can be achieved in various ways, including oral, buccal, rectal, parenteral, intraperitoneal, intradermal, transdermal, intratracheal, etc., administration.

In pharmaceutical dosage forms, the compounds may be administered in the form of their pharmaceutically acceptable salts, or they may also be used alone or in appropriate association, as well as in combination, with other pharmaceutically active compounds. The following methods and excipients are merely exemplary and are in no way limiting.

For oral preparations, the compounds can be used alone or in combination with appropriate additives to make tablets, powders, granules or capsules. Examples of additives are conventional additives, such as lactose, mannitol, corn starch or potato starch; binders, such as crystalline cellulose, cellulose derivatives, acacia, corn starch or gelatins; disintegrators, such as corn starch, potato starch or sodium carboxymethylcellulose; lubricants, such as talc or magnesium stearate; and if desired, diluents, buffering agents, moistening agents, preservatives and flavoring agents.

The compounds of the invention can be formulated into preparations for injection by dissolving, suspending or emulsifying them in an aqueous or nonaqueous solvent, such as vegetable or other similar oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol. If desired, conventional additives such as solubilizers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives may also be added. The concentration of therapeutically active compound in the formulation may vary from about 0.5-100 wt.%.

The compounds can be utilized in aerosol formulation to be administered via inhalation. The compounds of the present invention can be formulated into pressurized acceptable propellants such as dichlorodifluoromethane, propane, nitrogen and the like.

Furthermore, the compounds can be made into suppositories by mixing with a variety of bases such as emulsifying bases or water-soluble bases. The compounds of the present invention can be administered rectally via a suppository. The suppository can include vehicles such as cocoa butter, carbowaxes and polyethylene glycols, which melt at body temperature, yet are solidified at room temperature.

Unit dosage forms for oral or rectal administration such as syrups, elixirs, and suspensions may be provided wherein each dosage unit (*e.g.*, a teaspoonful, tablespoonful, tablet or suppository) contains a predetermined amount of the composition containing one or more inhibitors. Similarly, unit dosage forms for injection or intravenous administration may comprise the inhibitor(s) in a composition as a solution in sterile water, normal saline or another pharmaceutically acceptable carrier.

The pharmaceutically acceptable excipients, such as vehicles, adjuvants, carriers or diluents, are readily available to the public. Moreover, pharmaceutically acceptable auxiliary substances, such as pH adjusting and buffering agents, tonicity adjusting agents, stabilizers, wetting agents and the like, are readily available to the public.

Compounds for use in the method of the invention may also be small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate compounds comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate compounds often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate compounds are also found among biomolecules including, but not limited to: peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or combinations thereof.

The compounds are added to a host in a physiologically acceptable carrier, at a dosage from 5 mg to 1400 mg, more usually from 100 mg to 1000 mg, preferably 500 to 700 for a dose of 0.5 to 20 mg/kg weight. The dosage for compounds suppressing thrombin response is elected so that the PAR1 and PAR4 activity is reduced by 10 to 80%, more preferably 20 to 70% and even more preferably 25-50%. The dosage for compounds inhibiting the activity of PAR1 and PAR4 is elected so that the ability of platelets to respond to thrombin is reduced by about 20 to 80%, preferably 40 to 50%.

Platelet activation may be induced by a number of biological phenomenon, including injury, response to certain compounds, etc. The subject compositions will generally be administered daily, in an amount to provide at least about a 50 to 100%, more preferably 75-95%, even more preferably 80-90% decrease in platelet activation. Generally, the total daily dosage will be at least about 10 mg, usually at least about 400 mg to 500 mg,

preferably about 700 mg, and not more than about 1500 mg, usually not more than about 1000 mg. The amount may vary with the general health of the patient, the response of the patient to the drug, whether the composition is used by itself or in combination with other drugs, and the like. Daily administrations may be one or more times, usually not more than about four times, particularly depending upon the level of drug which is administered.

Administration of the compounds of the invention is particularly useful in the treatment of diseases such as myocardial infarction, stroke, pulmonary embolism, deep vein thrombosis, peripheral arterial occlusion, and other blood thromboses. Inhibition of platelet activation in such disorders may allow localized treatment at the site of the clotting, thus eliminating some of the more unpleasant side effects of systemic treatment, *e.g.* hemorrhage.

EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make receptor proteins and sequences encoding such proteins and carry out the methodology for finding such DNA sequences and proteins, and are not intended to limit the scope of what is regarded as the invention. Efforts have been made to insure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight; temperature is in degrees centigrade; and pressure is at or near atmospheric.

EXAMPLE 1: GENERATION AND CHARACTERIZATION OF PAR POLYCLONAL ANTIBODIES

The synthetic peptides GGDDSTPSILPAPRGYPGQVC (PAR4 amino acids 34-55, SEQ ID NO:1), AKPTLPIKTFRGAPPNSFEEFPFSALEGC (PAR3 amino acids 31-58 plus carboxyl glycine-cysteine, SEQ ID NO:2) and NATLDPRSFLLRNPNDKYEPFWEDEEGC (PAR1 amino acids 35-61 plus carboxyl glycine-cysteine, SEQ ID NO:3) were used to generate polyclonal antisera in rabbits. Ig was purified by Protein-A affinity chromatography to generate the PAR4 IgG, PAR3 IgG and PAR1 IgG preparations used in this study. Binding of PAR1 PAR3, PAR4 immune IgGs and PAR4 pre-immune IgG to each receptor

was tested on COS cells transiently expressing FLAG epitope- tagged receptors using an enzyme-linked immunosorbent assay (ELISA) as previously described (H. Ishihara et al., *Blood*, 91(11):4152-4157 (1998); K. Ishii et al., *J. Biol. Chem.*, 268:9780-6 (1993). cDNA for an epitope-tagged human PAR4 analogous to FLAG epitope-tagged PAR1 and was constructed as previously described such that the FLAG epitope was fused to amino acid 22 in PAR4 to yield the following sequence:

...DYKDDDDVE/TPSVYE...

where / indicates the junction with the PAR4 sequence.

EXAMPLE 2: EXPRESSION OF PAR mRNAS IN PLATELETS AND OTHER BLOOD CELLS

A competitive RT-PCR assay was developed to assess expression of mRNAs encoding the known PARs. First, a competitor RNA (cRNA) was generated for each PAR mRNA. Each cRNA was identical to the sequence of native mRNA to be reverse transcribed and amplified by PCR except for mutation of a single restriction endonuclease site; digestion with the cognate restriction endonuclease thus allowed differentiation of PCR products generated from cRNA vs. mRNA. Varying amounts of competitor cRNAs were added to 200ng of total cellular RNA and the mixtures were reverse transcribed and amplified by PCR using PAR-specific primers. In the absence of reverse transcription, no products were amplified. The products of reactions that included only native mRNA were completely cleaved by the appropriate restriction endonuclease while the products of reactions that included only cRNA remained undigested. The addition of increasing amounts of competitor cRNA to total cellular RNA prior to RT-PCR and comparison of the intensity of the bands obtained after restriction endonuclease digestion of the resulting PCR products allowed estimation of the amount of PAR mRNA in each sample.

Dami cells were grown in suspension in RPMI with 10% fetal bovine serum. Platelets were separated from human blood as previously described. Platelet preparations contained less than 0.1% leukocytes as assessed by light microscopic analysis. A discontinuous Percoll gradient was used to separate monocytes plus lymphocytes from neutrophils according to the manufacturer's instructions (Pharmacia). The monocyte/lymphocyte preparations contained less than 0.1% neutrophils and the neutrophil preparations contained less than 0.1% monocytes or lymphocytes. Total RNA was prepared

from all cells using Trizol (GIBCO), treated with DNAse I (Boehringer-Mannheim), and quantified by OD_{260}.

Each receptor cDNA was mutated so as to ablate an endogenous restriction endonuclease site. Sites were mutated by digesting cDNA encoding the receptor with the selected endonuclease, exposing the digested plasmid to T4 DNA polymerase then religating. Each of these mutant cDNAs was subcloned into Bluescript (KS- or SK-, Stratagene) such that sense cRNA could be transcribed *in vitro* using T7 RNA polymerase. Competitor cRNAs so generated were added to total cell RNA prior to reverse transcription (RT) reactions. RT reactions were performed at 42°C using 200 ng of total RNA with varying amounts of competitor cRNA in a 10 μl reaction volume using a commercial kit (GIBCO) and receptor specific primers (see below). 2 μl of the RT product was then subjected to PCR amplification in a 50 μl volume containing a final concentration of 2 μM primers and 5U of Taq polymerase (GIBCO). Reaction conditions were as follows: 94°C for 4 min, 72°C for 1 min with addition of Taq; then 94°C for 45 sec, 55°C for 1 min, 72°C for 1 min for 30-36 cycles (see below); then 72°C for 8 min. For each sample, a preliminary experiment was performed in which the number of amplification cycles was varied to determine the cycle number over which specific product was amplified and a cycle number in the middle of the dynamic range was chosen. For each analysis, the number of cycles chosen for measurement of PAR1, PAR2, PAR3, and PAR4 mRNA levels, respectively, in RNA from the various sources was:

	PAR1	PAR2	PAR3	PAR4
Platelets:	31	36	36	36
Neutrophils:	36	27	31	36
Monocytes/ Lymphocytes	31	31	33	36
Dami cells	30	32	32	33

Preliminary experiments also established the range of concentrations of competitor RNA to be added to each sample.

The primers used for RT and PCR of each receptor and the restriction endonuclease used to digest each PCR product were as follows. The convention for nucleotide numbering is that 1 = the A of the start methionine ATG.

PAR1, GenBank accession # M62424:

Primer for RT: TAG ACG TAC CTC TGG CAC TC (1148-1129; SEQ ID NO:4).
Sense strand primer for PCR: CAG TTT GGG TCT GAA TTG TGT CG (SEQ ID NO:5).
Anti-sense primer for PCR: TGC ACG AGC TTA TGC TGC TGA C (SEQ ID NO:6).
Resulting PCR product: 505-1096.
Mutated site: AgeI at position 596.

PAR2, GenBank accession # U34038:
Primer for RT: CTG CTC AGG CAA AAC ATC (699-682; SEQ ID NO:7).
Sense strand primer for PCR: TGG ATG AGT TTT CTG CAT CTG TCC.(SEQ ID NO:8)
Anti-sense primer for PCR: CGT GAT GTT CAG GGC AGG AAT G (SEQ ID NO:9).
Resulting PCR product: 182-672.
Mutated site: SfiI at position 342.

PAR3 GenBank accession #U92972:
Primer for RT: TGA TGT CTG GCT GAA CAA G (727-709; SEQ ID NO:10).
Sense strand primer for PCR: TCC CCT TTT CTG CCT TGG AAG.(SEQ ID NO:11)
Anti-sense primer for PCR: AAA CTG TTG CCC ACA CCA GTC CAC (SEQ ID NO:12).
Resulting PCR product: 152-664.
Mutated site: NcoI at position 251.

PAR4, GenBank accession #AF080214:
Primer for RT: TGA GTA GCT GGG ATT ACA G (1519-1501; SEQ ID NO:13).
Sense strand primer for PCR: AAC CTC TAT GGT GCC TAC GTG C (SEQ ID NO:14).
Anti-sense primer for PCR: CCA AGC CCA GCT AAT TTT TG (SEQ ID NO:15).
Resulting PCR product: 949-1490.
Mutated site: BamHI at position 1005.

Following PCR amplification, 10 μl of reaction product was digested overnight with the appropriate restriction endonuclease (AgeI, 2U at 25°C; SfiI, 20U at 50°C; NcoI, 10U at 37°C; or BamHI, 20U at 37°C). The products were then separated by 1.5%, agarose gel electrophoresis (Separide gel matrix, GIBCO) and visualized by ethidium bromide staining. The cRNA concentration at which the intensity of the cRNA-derived product (uncleaved

band) matched that of the endogenous mRNA-derived product (cleaved band) was used to estimate the quantity of each PAR mRNA in the original sample.

The summary of results of several studies of a competitive RT-PCR assay of RNA prepared from platelets, neutrophils and Dami cells is presented in Fig. 1. To validate the assay, Dami cells, a human cell line that express some megakaryocyte markers were analyzed first. Competitive RT PCR of Dami cell RNA yielded results that were concordant with Northern and protein analysis. For Northern analysis, 2 μg of poly(A)+ RNA isolated from Dami cells was electrophoresed on a denaturing formaldehyde agarose gel and transferred onto a supported nitrocellulose membrane (Schleicher & Schuell). PAR1 mRNA was detected with a 400 bp PstI/PvuII cDNA probe; PAR2 mRNA was detected with a 260 bp SfiI/BstEII cDNA probe; PAR3 mRNA was detected with a 610 bp KpnI/NsiI cDNA probe; PAR4 mRNA was detected using a 450 bp SacI/PstI cDNA probe using high stringency conditions. In all such assays, PAR1, PAR3, and PAR4 were detected in Dami cells while PAR2 was not (Fig. 1).

Competitive RT-PCR of platelet RNA revealed PAR1 mRNA to be present at approximately one attomole/200 ng total RNA. Assuming mRNA is 1% of total platelet RNA and an average mRNA size of 2kb, PAR1 mRNA represents one in three thousand platelet mRNAs. PAR4 mRNA was also readily detected in platelet RNA at 10-30% of PAR1 mRNA levels. By contrast, PAR3 mRNA was undetectable in human platelet RNA. PAR3 competitor cRNA added to platelet RNA was detectable at 0.001 attomole/200ng total RNA, suggesting that PAR3 mRNA was at least 1000 fold less abundant than PAR1 mRNA in these samples. PAR2 mRNA was not detected in platelet RNA from one individual and only 0.001 attomole/200ng was detected in the other. The latter may be due to trace contamination of the platelet preparation by neutrophils, which do express PAR2. Inability to detect significant PAR2 mRNA in platelets is consistent with the observation that the specific PAR2 agonist peptide SLIGKV is unable to activate human platelets.

The pattern of PAR mRNA expression in neutrophils and mononuclear cells was distinct from that seen in platelets, suggesting that contamination of platelet preparations by leukocytes did not significantly influence the PAR expression pattern detected in platelets. In particular, while virtually absent from platelets, substantial PAR2 mRNA was detected in RNA from both neutrophils and mononuclear cells. The relatively high PAR2 mRNA level in neutrophils is consistent with previous studies demonstrating neutrophil responses to

PAR2 activating peptide. In contrast to platelets, PAR3 mRNA was consistently detected at low levels in mononuclear cells. PAR4 mRNA was also found in mononuclear cell preparations but was not detected in neutrophils. These results demonstrate the presence of mRNA encoding PAR1 and PAR4, but not PAR2 or PAR3, in human platelets.

EXAMPLE 3: Expression of PAR proteins on the surface of human platelets

IgG was purified from rabbit anti-peptide antisera directed against the amino terminal exodomains of PAR1, PAR3, or PAR4. To characterize the ability of each IgG preparation to recognize native PARs and to assess cross-reactivity, antibody binding to the surface of receptor-expressing COS cells was measured (Fig. 2). Each IgG preparation bound to the surface of cells expressing the appropriate receptor without significant cross-reactivity.

The IgG preparations were then used for flow cytometric analysis of human platelets (Figs. 3A - 3D). Washed platelets were fixed in paraformaldehyde for 20 minutes at 40°C, washed three times with platelet buffer (20mM Tris-HCl pH 7.4, 140mM NaCl, 2.5mM KC, 1mM $MgCl_2$, 1mg/ml glucose, 0.5% BSA), then incubated with primary IgG in platelet buffer at 40°C for 1 hour. PAR1 and PAR3 IgG were used at 10 μg/ml and PAR4 IgG at 100 μg/ml. Platelets were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Molecular Probes) at 4 μg/ml for 0.5 hour. Platelets were then washed three times and analyzed by flow cytometry. Some fixed platelet samples were exposed to 30 nM thrombin at 37°C prior to incubation with primary antibody. Dami cells were treated like platelets for flow cytometry.

Significant surface binding was detected with PAR1 IgG compared with preimmune IgG (Fig. 3A). A similar increase in platelet surface binding was detected with PAR4 IgG vs. preincubation the peptide antigen to preimmune IgG (Fig. 3C). Preincubation of PAR4 with the peptide antigen to which it was raised abolished this increase (Figs. 3C and D). Moreover, the epitope to which the PAR4 antiserum was raised spans PAR4's thrombin cleavage site, and treatment of platelets with thrombin indeed abolished PAR4 IgG binding (Figs. 3C and D). These data strongly suggest that PAR1 and PAR4 are expressed on the surface of human platelets.

PAR3 immune IgG showed no specific binding to human platelets. To determine whether this antibody could detect PAR3 expressed at "natural" levels, this experiment was

repeated with Dami cells (Figs. 4A - 4D), which had been shown by Northern blot to express PAR3 mRNA. As was observed for platelets, a significant increase in fluorescence was seen using PAR1 and PAR4 antibodies (Figs. 4A, 4C and 4D). In contrast to platelets, a significant increase in fluorescence was also observed with PAR3 antibody. This result is consistent with the presence of PAR3 mRNA in Dami cells by RT-PCR and Northern blot analysis. It further suggests that the absence of detectable PAR3 protein on the surface of human platelets is not due to insensitivity of the assay. These results are consistent with the analysis of platelet RNA and confirm the presence of PAR1 and PAR4 but not PAR3 on the surface of human platelets.

EXAMPLE 4: ACTIVATION OF HUMAN PLATELETS BY PAR1 AND PAR4 TETHERED LIGAND PEPTIDES

Synthetic peptides that mimic the tethered ligands of PAR1 and PAR2 function as agonists for their respective receptors and have been used as pharmacological tools to probe the function of these receptors in various cell types. Unfortunately, the cognate peptide for PAR3 appears to be insufficiently avid to function as a free ligand. Peptides mimicking the tethered ligand for PAR4 can function as an agonist for that receptor, albeit at a concentration higher than that seen for the PAR1 and PAR2 peptides and their cognate receptors. To assist in the interpretation of experiments using the PAR1 and PAR4 tethered ligand peptides, the ability to activate PAR1 and PAR4 heterologously expressed in *Xenopus* oocytes (a very sensitive assay system) was determined (Fig. 5). Agonist-triggered calcium mobilization was used an index of receptor activation. No responses were detected in oocytes expressing neither receptor. Both the human PAR4 peptide GYPGQV and the mouse PAR4 peptide GYPGKF activated oocytes expressing human PAR4, but with an EC_{50} roughly two orders of magnitude higher than that of SFLLRN for PAR1 activation (Fig. 5). SFLLRN showed no activity at PAR4. At 500μM, the PAR4 peptide GYPGKF did show minimal activity at PAR1. However, PAR1 is overexpressed in the oocytes and the sensitivity for detection of PAR1 activation in the oocyte assay is 10-100 fold greater than in platelets; it is likely that PAR1 activation at 500μM GYPGKF is unimportant in the platelet studies described below.

The PAR1 peptide SFLLRN and the PAR4 peptides GYPGKF and GYPGQV all activated human platelets (Fig. 6A). Both PAR4 peptides were considerably less potent than

the PAR1 peptide for activating human platelets. GYPGKF was slightly more potent than GYPGQV (Fig. 6A and data not shown).

These data are consistent with the relative potencies with the activation of the receptors by their respective peptides in the oocyte system (Fig. 5). In short, FLAG epitope-tagged PAR4 cDNA was subcloned into pFROG[3] and signaling studies performed in *Xenopus* oocytes as described. J. A. Williams et al., *PNAS USA* 85:4939-43 (1998). 2.0 ng of PAR4 cRNA and 25 ng of PAR1 cRNA was injected per oocyte.

Incubation of PGE_1-treated platelets with SFLLRN rendered them refractory to subsequent stimulation by SFLLRN but did not effect responsiveness to GYPGKF (Fig. 6B). Conversely, incubation with GYPGKF rendered platelets refractory to subsequent stimulation by GYPGKF but did not effect responsiveness to SFLLRN (Fig. 6C). These results suggest that activation of either PAR1 or PAR4 with their cognate peptide agonists is sufficient to activate human platelets.

EXAMPLE 5: PAR1 AND PAR4 ANTIBODIES INHIBIT THROMBIN CLEAVAGE OF THEIR RESPECTIVE RECEPTORS

Toward defining the necessary roles of PAR1 and PAR4 in platelet activation by thrombin, blocking antibodies were developed. The previously described PAR1 antibody raised against PAR1's hirudin-like domain) is predicted to inhibit thrombin cleavage of PAR1's amino terminal exodomain by disrupting binding to thrombin's anion-binding exosite. No analogous hirudin-like domain was apparent in the sequence of PAR4's amino terminal exodomain. To obtain an antibody that would inhibit thrombin cleavage of PAR4, antiserum was raised to a peptide that represented sequence spanning PAR4's thrombin cleavage site. This antiserum specifically recognized PAR4. To test the ability of the PAR1 and PAR4 antibodies to block thrombin cleavage of PAR1 and PAR4, Rat I fibroblasts expressing FLAG epitope-tagged PAR1 and PAR4 were preincubated with antibody then exposed to thrombin. Receptor cleavage by thrombin was measured as loss of FLAG epitope from the cell surface. PAR1 cleavage was markedly inhibited by PAR1 antibody but not by PAR4 antibody. Conversely, PAR4 cleavage was markedly inhibited by PAR4 antibody but not by PAR1 antibody (Fig. 7). These data suggested that the PAR1 and PAR4 antibody should selectively attenuate thrombin signaling via PAR1 and PAR4, respectively.

EXAMPLE 6: INHIBITION OF THROMBIN SIGNALING BY PAR1 AND PAR4 ANTIBODIES AND BY A PAR1 ANTAGONIST.

A *Par 1*$^{-/-}$ mouse lung fibroblast cell line that showed no thrombin signaling was used to generate stable cell lines expressing FLAG epitope-tagged PAR1 and PAR4. Increases in cytoplasmic calcium in response to thrombin were measured using the calcium-sensitive dye Fura-2 as previously described. A. J. Connolly, et al., *Nature*, 381:516-519 (1996). Because no thrombin responses were detectable in untransfected *Par1*$^{-/-}$ fibroblasts, signaling in the transfected cells could be attributed to the transfected receptor. In the PAR1-expressing cell line, increases in cytoplasmic calcium were reliably elicited by thrombin at concentrations as low as 10 pM (Fig. 8A). PAR4 IgG had no inhibitory effect even on these threshold responses (Figs. 8A and 8B). PAR1 IgG markedly attenuated such signaling and non-immune antibody was without effect. Strikingly, the PAR1 antagonist BMS20026121 attenuated PAR1 signaling even at high thrombin concentrations (Figs. 8A and 8B). The peptide-based PAR1 antagonist BNIS200261 was synthesized as previously described in N. I. Bernatowicz, et al., *J. Med. Chem.* 39:4879-87 (1996). Responsiveness to lysophosphaticlic acid was unaffected by the antagonist (Figs. 8A and 8B), suggesting that its inhibitory effect was specific.

In the PAR4-expressing cell line, increases in cytoplasmic calcium were reliably triggered at 0.1 nM thrombin (Fig. 8B). PAR4 IgG blocked such responses but had no effect on responses to GYPGKF, consistent with the antibody's acting by preventing receptor cleavage by thrombin. PAR4 preimmune IgG, PAR1 IgG, and PAR1 antagonist (100 μM) failed to inhibit PAR4 signaling even at low thrombin concentrations (Fig. 8B).

Taken together, these results demonstrate that PAR4 IgG blocked PAR4 but not PAR1 signaling in response to thrombin, and PAR1 IgG attenuated PAR1 but not PAR4 signaling in response to thrombin. Moreover, the PAR1 antagonist BMS 200261 was remarkably effective in blocking PAR1 signaling but was without effect on PAR4. Lastly, receptor desensitization studies with SFLLRN in PAR1 and PAR4 transfected fibroblasts (not shown) and in human platelets (Figs. 6A - 6C) demonstrated that prolonged incubation with SFLLRN was an additional means of attenuating PAR1 signaling.

EXAMPLE 7: INHIBITION OF PLATELET AGGREGATION BY INHIBITING PAR1 AND PAR4 SIGNALING.

The contribution of PAR1 and PAR4 signaling to thrombin activation of human platelets was tested to determine each molecule's relative contribution to the process. Platelets were prepared using whole blood from physiologically normal volunteers and aggregation and secretion were measured. For desensitization studies, SFLLRN (100 μM) or GYPGKF (500 μM) were added to platelets resuspended from the first platelet pellet and platelets were incubated at room temperature for 30 minutes without stirring prior to repeat centrifugation. For functional studies with PAR1 or PAR4 antibody, platelets were incubated with antibody or preimmune IgG for 60 minutes prior to measurement of secretion and aggregation. PAR1 antagonist was added to stirring platelets 1-2 minutes prior to the addition of thrombin or other agonists.

By itself, PAR4 IgG had no effect on platelet aggregation even at low (1nM) thrombin responses (Figs. 9A - 9E). By contrast, PAR1 IgG or PAR1 antagonist markedly inhibited platelet aggregation in response to 1 nM thrombin, as did prior desensitization of platelets with the PAR1 agonist SFLLRN. None of these maneuvers inhibited platelet aggregation in response to GYPGKF or submaximal concentrations of ADP. These data suggest that PAR1 is the major mediator of platelet activation at low concentrations of thrombin.

In contrast to the case at 1 nM thrombin, at 30 nM thrombin, inhibition of PAR1 signaling by either PAR1 IgG, antagonist, or SFLLRN desensitization only slowed aggregation slightly such that shape change became detectable (see 0-30 second portions of the aggregation curves in Figures 9B, 9C, and 9D). Otherwise, these maneuvers were without inhibitory effect. Inhibition of PAR4 signaling with PAR4 IgG was similarly ineffective (Fig. 9B).

Strikingly, when signaling via PAR1 and PAR4 were blocked simultaneously, aggregation in response to even high concentrations of thrombin was virtually abolished (Figs. 9A - 9E). Such synergy was seen regardless of the means by which PAR1 was blocked (desensitization, PAR1 IgG, or antagonist). PAR4 preimmune concentrations as high as 30 nM. They also suggest that PAR1 is necessary for rapid platelet activation by thrombin even at high thrombin concentrations.

The instant invention is shown and described herein in what is considered to be the most practical, and preferred embodiments. It is recognized, however, that departures may be made therefrom, which are within the scope of the invention, and that obvious modifications will occur to one skilled in the art upon reading this disclosure, and thus the invention is limited only by the appended claims.

CLAIMS

That which is claimed is:

1. A method for affecting platelet activation, comprising:

administering an effective amount of a first compound characterized by its ability to specifically modulate PAR1 activity; and

administering an effective amount of a second compound characterized by its ability to specifically modulate PAR4 activity.

2. The method of claim 1, wherein the first compound specifically inhibits PAR1 activity and the second compound specifically inhibits PAR4 activity.

3. The method of claim 1, wherein the first compound specifically activates PAR1 activity and the second compound specifically activates PAR4 activity.

4. The methods of claims 1, 2 and 3 wherein the first and second compounds are administered simultaneously to a subject.

5. A pharmaceutical composition comprising:

a therapeutically effective amount of a first compound characterized by its ability to specifically modulate PAR1 activity; and

a therapeutically effective amount of a second compound characterized by its ability to specifically modulate PAR4 activity.

6. The pharmaceutical composition of claim 5, wherein the first compound specifically inhibits PAR1 activity and the second compound specifically inhibits PAR4 activity.

7. The pharmaceutical composition of claim 6, wherein the first compound is PAR1 antagonist BMS 200261 and the second compound is a PAR4 antibody directed against a thrombin binding site of PAR4.

8. The pharmaceutical composition of claim 6, wherein the first compound is the desensitizing peptide SFLLRN and the second compound is the desensitizing peptide GYPGKF.

9. The pharmaceutical composition of claim 5, wherein the first compound specifically activates PAR1 activity and the second compound specifically activates PAR4 activity.

10. The pharmaceutical composition of claim 9, wherein the first compound is a PAR1 agonist and the second compound is a PAR4 agonist.

11. A PAR4 antibody directed against all or part of a thrombin binding site of PAR4.

12. The antibody of claim 11, wherein the antibody is directed against the sequence GGDDSTPSILPAPRGYPGQVC.

8. The pharmaceutical composition of claim 6, wherein the first compound is the desensitizing peptide SFLLRN and the second compound is the desensitizing peptide GYPGKF.

9. The pharmaceutical composition of claim 5, wherein the first compound specifically activates PAR1 activity and the second compound specifically activates PAR4 activity.

10. The pharmaceutical composition of claim 9, wherein the first compound is a PAR1 agonist and the second compound is a PAR4 agonist.

11. A PAR4 antibody directed against all or part of a thrombin binding site of PAR4.

12. The antibody of claim 11, wherein the antibody is directed against the sequence GGDDSTPSILPAPRGYPGQVC.

	PAR1	PAR2	PAR3	PAR4
	(attomoles/200 ng total RNA)			
Platelets (1)	1	0.001	<0.001	0.3
(2)	0.3–1	<0.001	<0.001	0.1
Dami cells	10	0.1	1–10	10
Neutrophils	0.01	3	0.03–0.1	<0.01
Monocytes + Lymphocytes	1	0.3	0.03–0.1	0.3

Figure 1

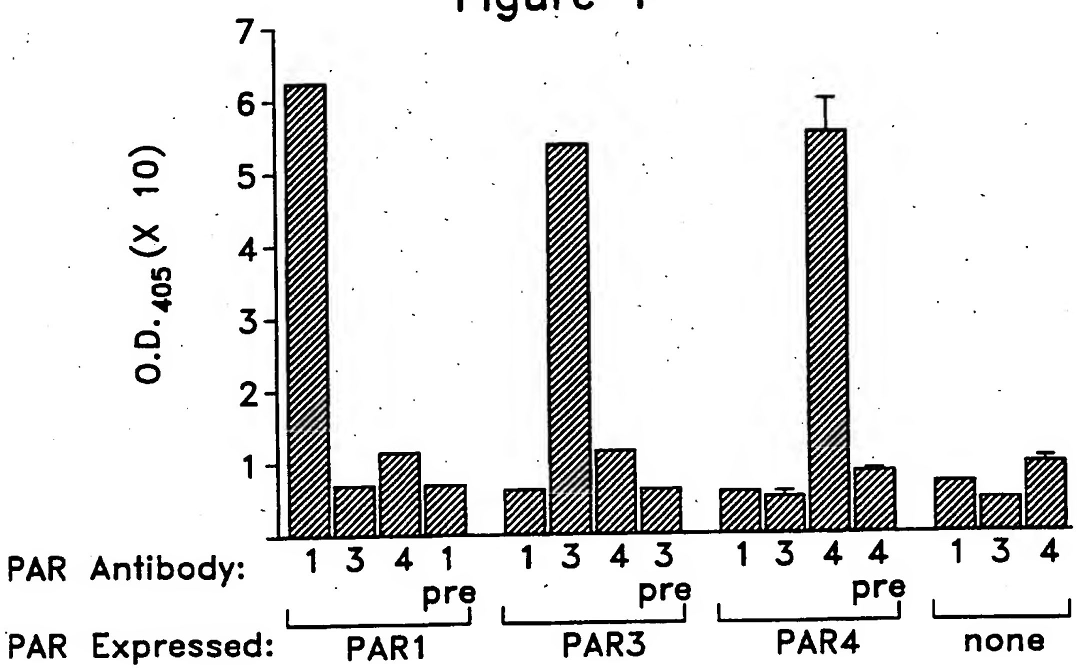


Figure 2

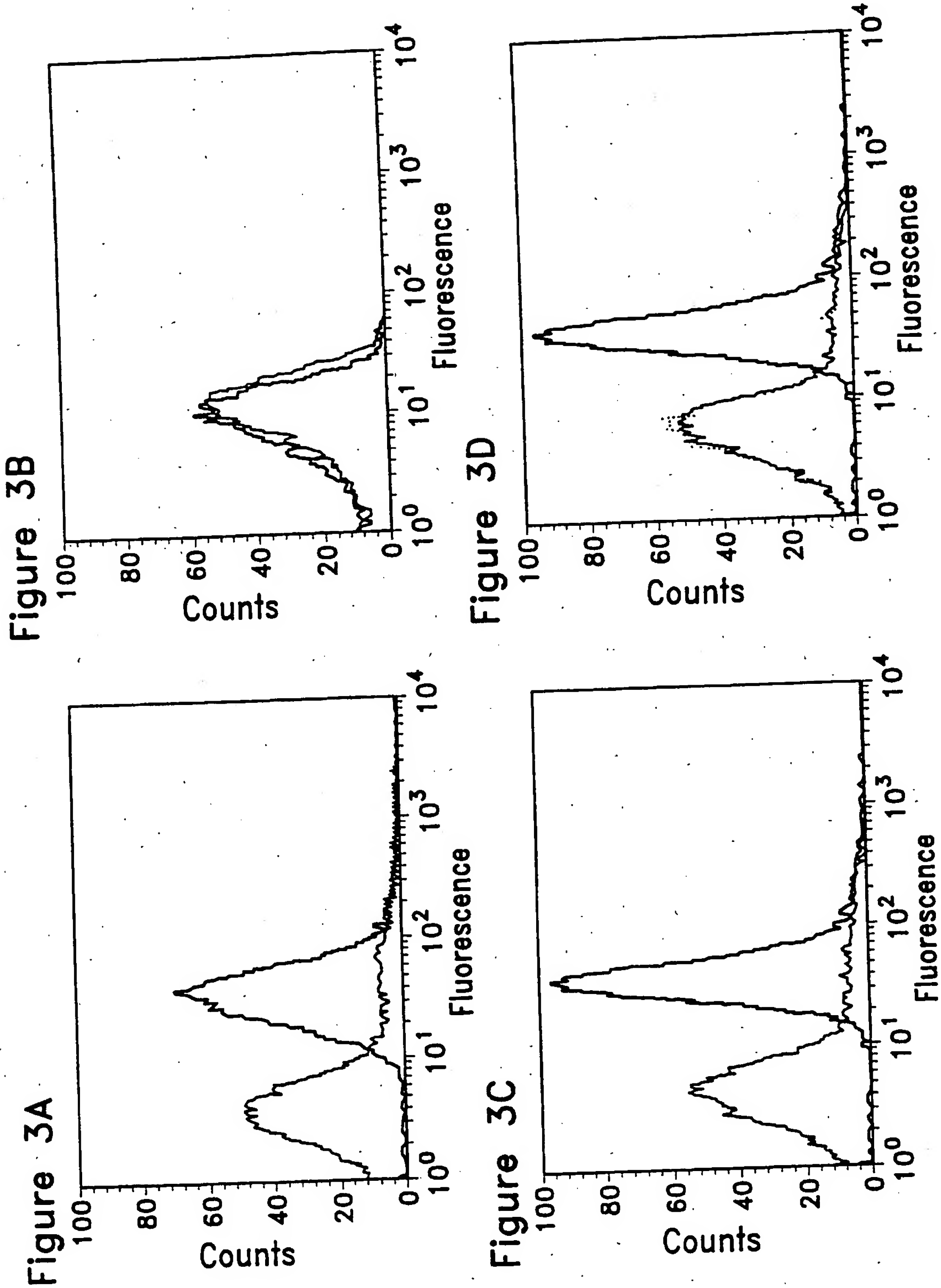


Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 4A

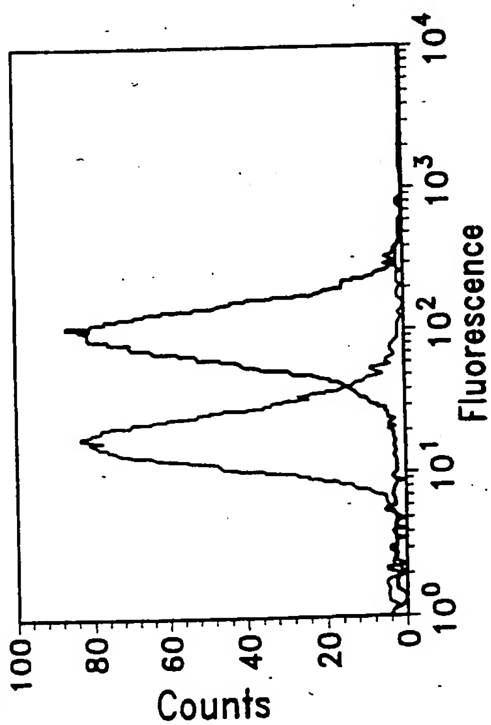


Figure 4B

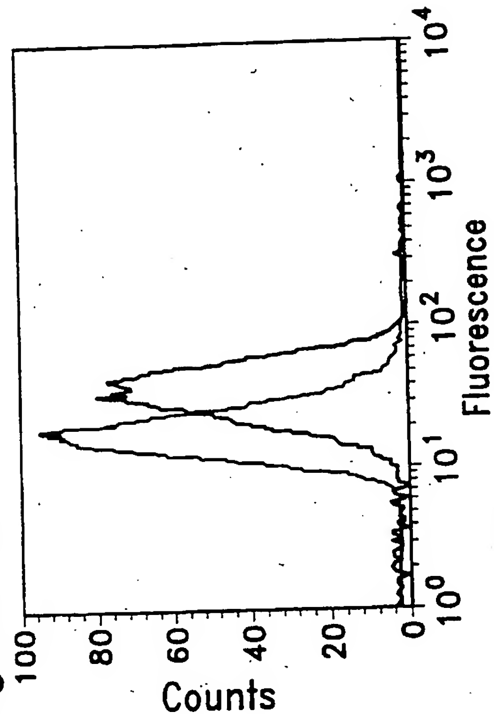


Figure 4C

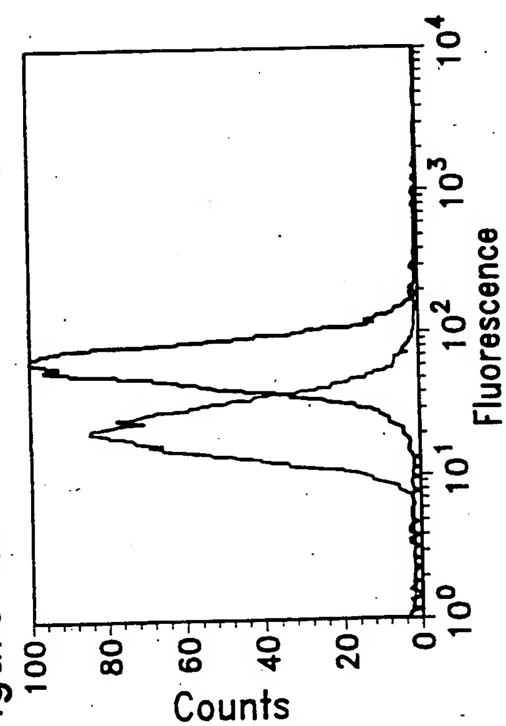


Figure 4D

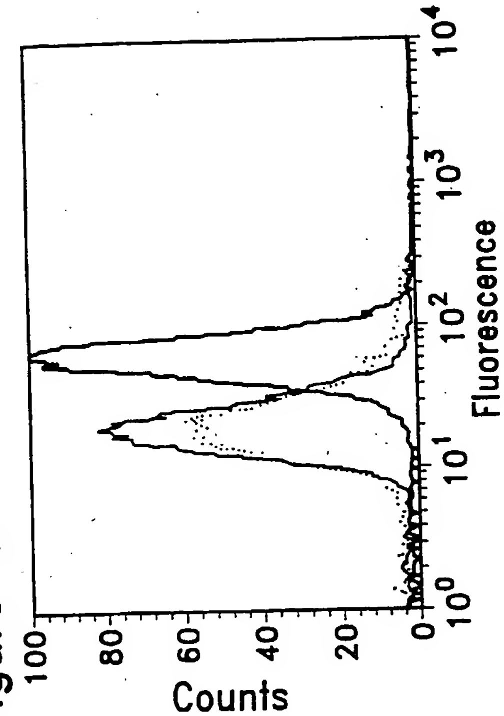
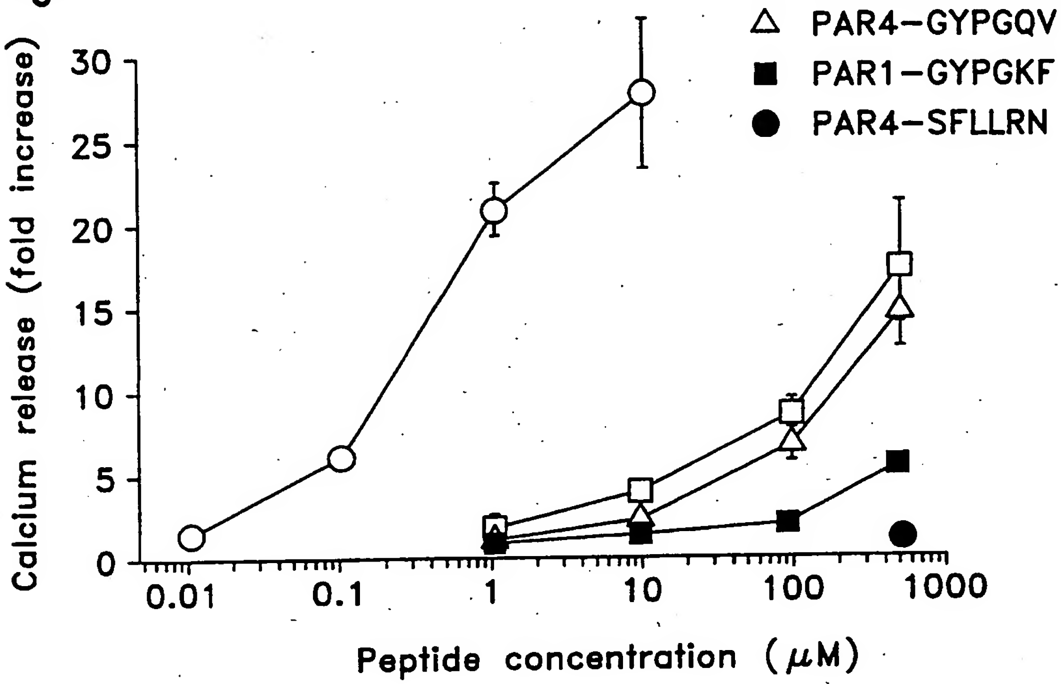


Figure 5



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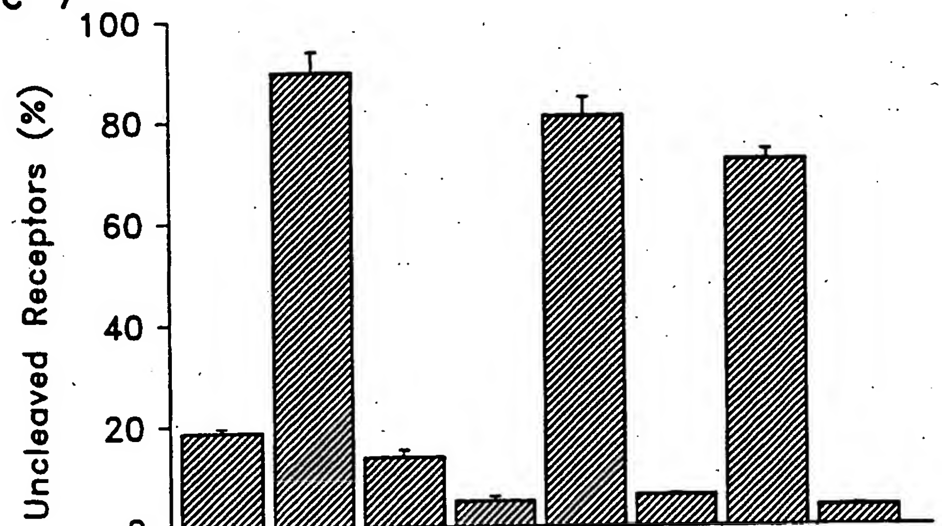

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Antibody	–	P1	P4	–	P1	–	P4	P1
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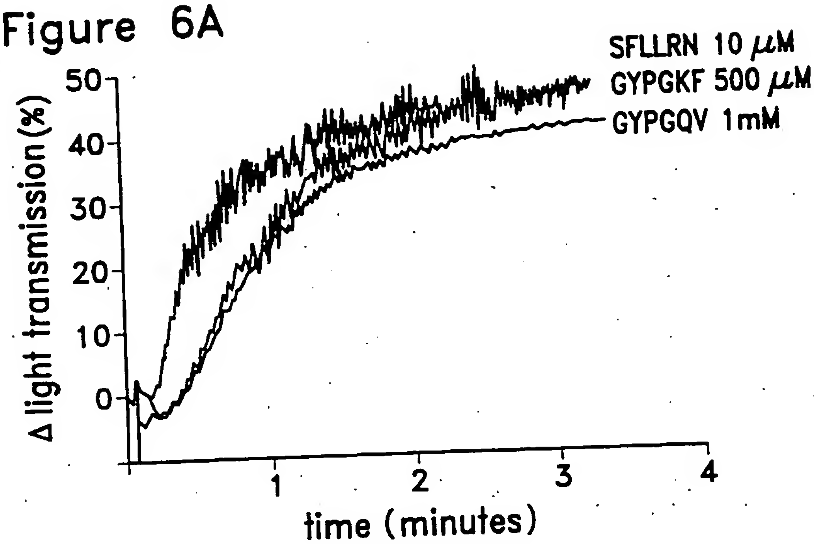

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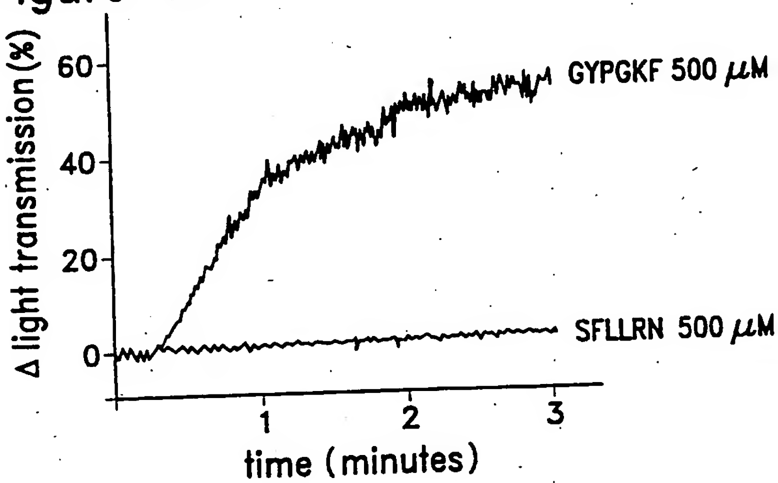

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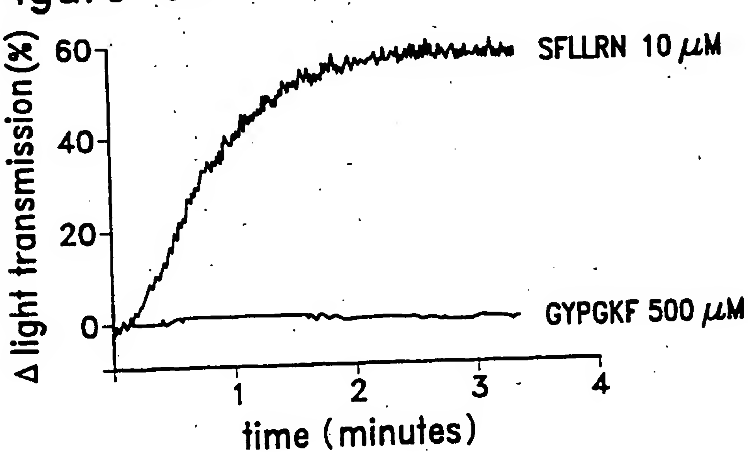


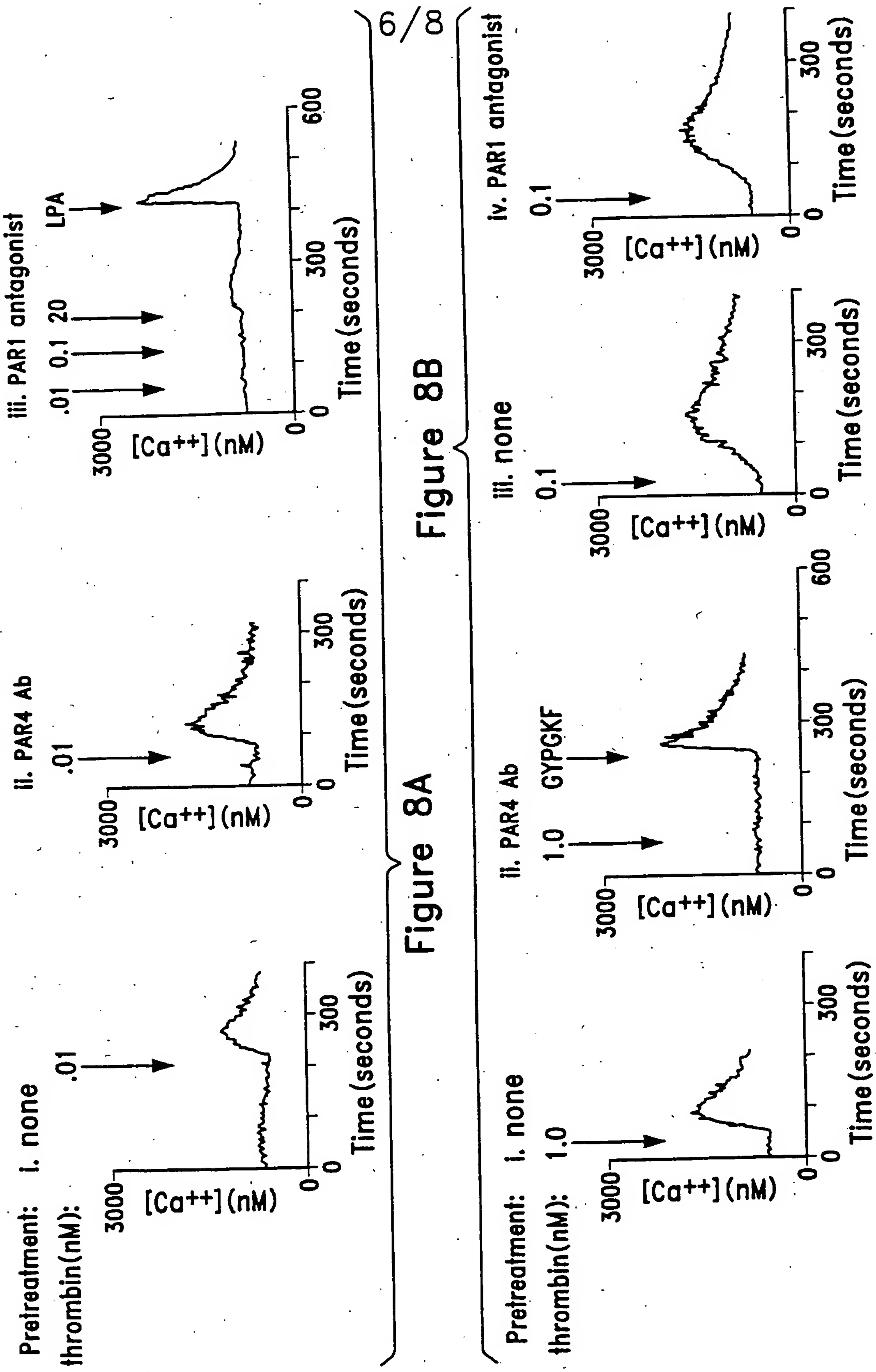


Figure 8A

Figure 8B

Figure 9A

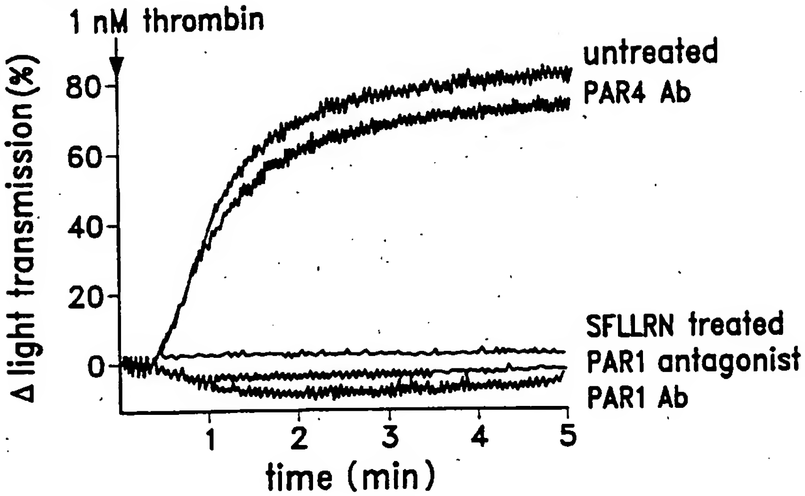


Figure 9B

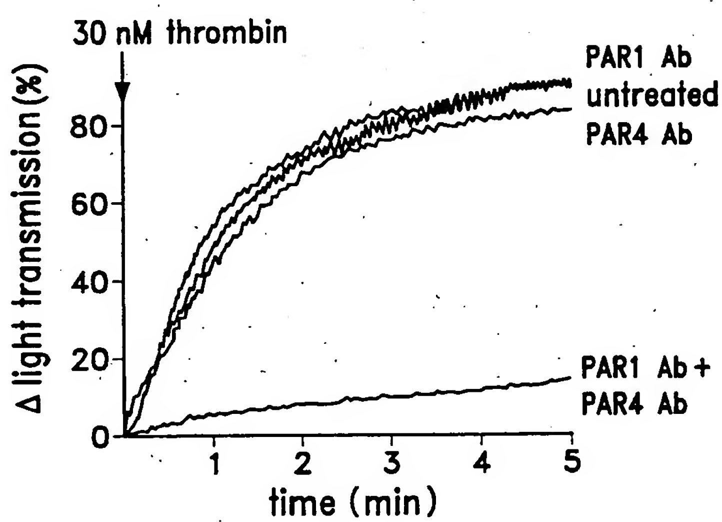
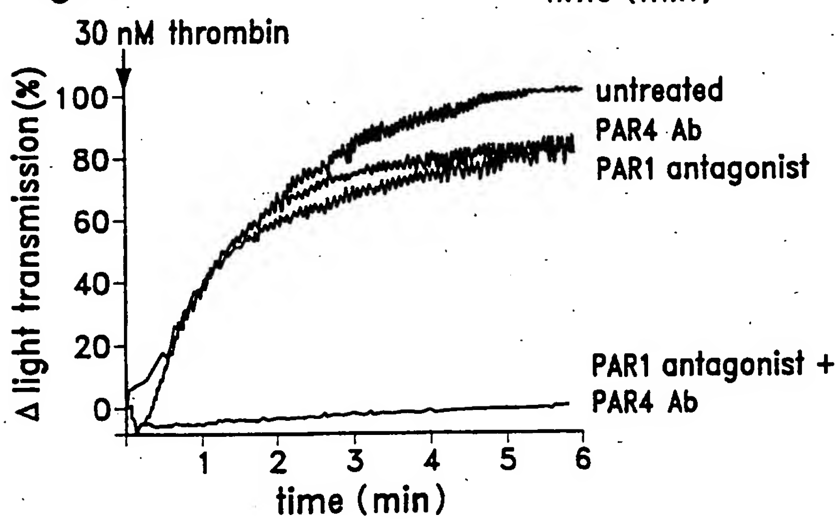


Figure 9C



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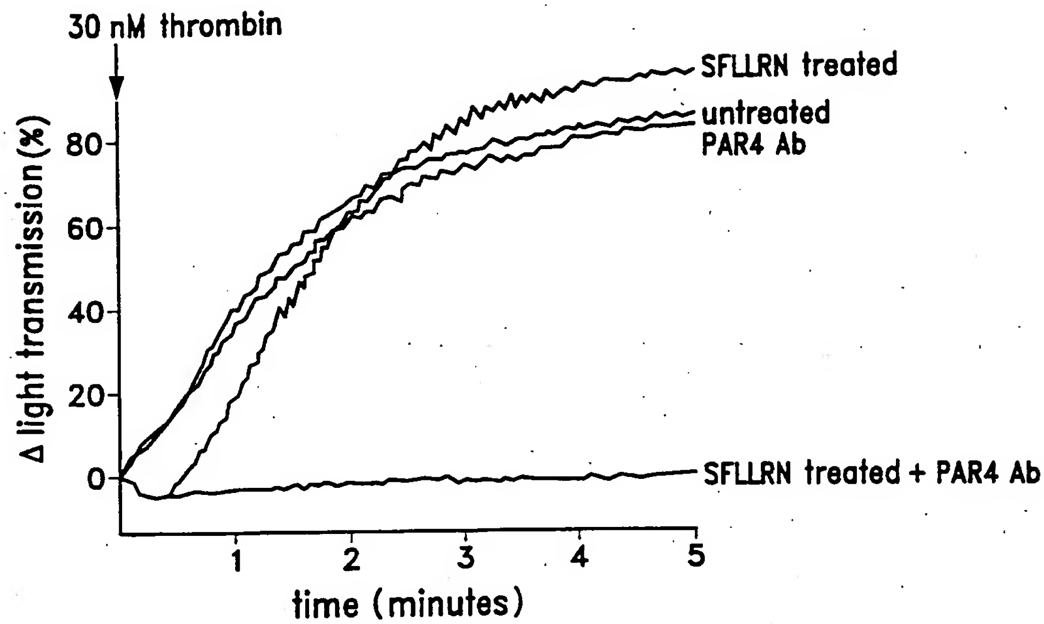

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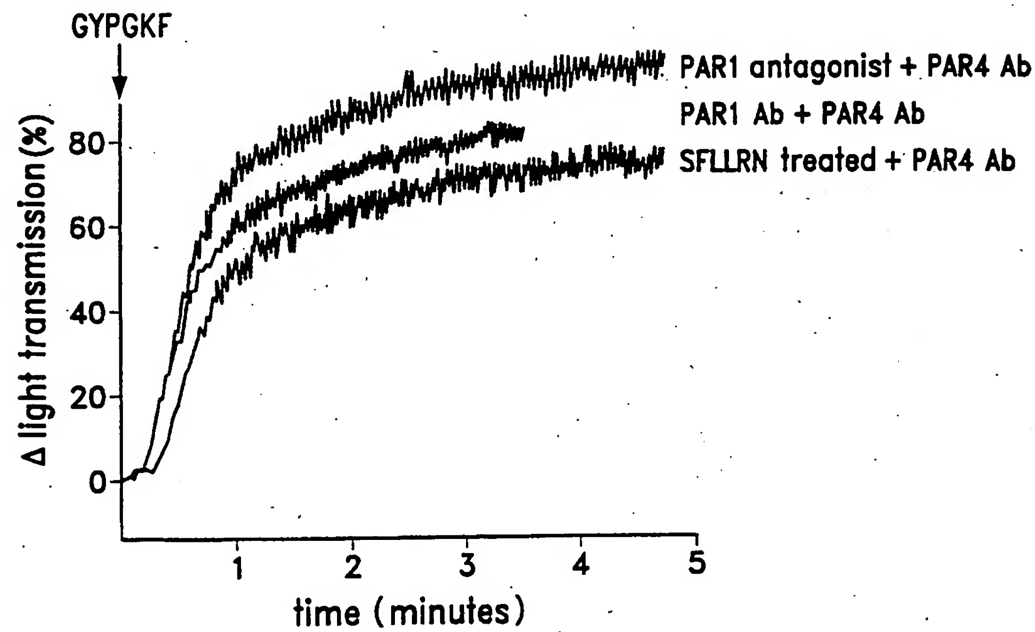

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Mark Kahn

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19158

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/02, 38/08, 38/04; C07K 16/18, 16/28
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/2, 17; 530/387.1, 388.1, 388.22, 388.7, 388.85, 389.1, 389.6, 387.9

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y,P	DERY et al. Proteinase-activated receptors: a growing family of heptahelical receptors for thrombin, trypsin and tryptase. Biochem. Soc. Transactions. 1999. Vol. 27, pages 246-254, especially page 249.	1-3, 5 and 6
Y	KHAN et al. A dual thrombin receptor system for platelet activation. Nature. 13 August 1998. Vol. 394, pages 690-694. See entire document.	1-3, 5, 6 and 8-10
Y	BERNATOWICZ et al. Development of potent thrombin receptor antagonist peptides. J. Med. Chem. 1996. Vol. 39, pages 4879-4887. See entire document.	1-3 and 5-7

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 NOVEMBER 1999	23 DEC 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Anne L. Holleran for ANNE L. HOLLERAN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	KAHN et al. Protease-activated receptors 1 and 4 mediate activation of human platelets by thrombin. J. Clin. Invest. March 1999, Vol. 103, pages 879-887, especially pages 881 and 883. See abstract and Figure 4.	1, 2, 11, 12 --- 5, 6, 8-10
A	SCARBOROUGH et al. Tethered ligand agonist peptides. J. Biol. Chem. 05 July 1992. Vol. 267, no.19, pages 13146-13149. See entire document.	1-3 and 5-12
A,P	HOLLENBERG et al. Protease-activated receptors: PAR4 and counting: how long is the course? Trends in Pharmacological Sciences. July 1999. Vol. 20, pages 271-273. See entire document.	1-3 and 5-12
A,P	KAWABATA et al. Modulation by protease-activated receptors of the rat duodenal motility in vitro: Possible mechanisms underlying the evoked contraction and relaxation. British J. Pharmacol. 1999. Vol. 128, pages 865-872. See entire document.	1-3 and 5-12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19158

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/19158

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

514/2, 17; 530/387.1, 388.1, 388.22, 388.7, 388.85, 389.1, 389.6, 387.9

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

REGISTRY, MEDLINE, EMBASE, BIOSIS, CAPLUS, US PATENTS (EAST/BRS)
search terms: bms2002621, bms 20026121, sfllrn, gypgkf, par 1, par 4, platelet activat?, protease activated receptor 1, protease activated receptor 4